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Epidemiologic and Surveillance Studies on Avian Influenza in Live-Bird Markets in New York and New Jersey, 2001

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SUMMARY. In 2001, all 109 retail live-bird markets (LBMs) in New York and New Jersey were surveyed for the presence of avian influenza virus (AIV) by a real time reverse transcriptase/polymer chain reaction assay (RRT/PCR) and results compared to virus isolation (VI) in embryonating chicken eggs. The RRT/PCR had a 91.9% sensitivity and 97.9% specificity in detecting presence of AIV at the market level. However, the sensitivity at the sample level is 65.87%. The RRT/PCR is a reliable method to identify AIV at the market level. In addition, a cross-sectional epidemiologic study of the LBMs showed that, during the past 12 months, markets that were open 7 days per week and those that also sold rabbits had the highest risk for being positive for AIV. Markets that were closed one or more days per week and those that performed daily cleaning and disinfecting had the lowest risk for being AIV positive.

RESUMEN. Epidemiología y Estudios de Vigilancia Epidemiológica de la Influenza Aviar en los Mercados de Aves en Pie de Nueva York y Nueva Jersey, 2001.

En el año 2001 se investigó la presencia del virus de la influenza aviar los 109 mercados de ventas de aves en pie de las ciudades de Nueva York y Nueva Jersey mediante el uso de la técnica de reacción en cadena por la polimerasa acoplada a trascripción reversa, en tiempo real (RRT-PCR), y se compararon estos resultados con los obtenidos mediante las técnicas de aislamiento viral en embrión de pollo. La técnica de RRT-PCR presentó una sensibilidad de un 91.9% y una especificidad de 97.9% para la detección de la presencia del virus en estos mercados. Sin embargo, la sensibilidad a nivel de muestras fue de un 65.87%. La prueba de RRT-PCR es un método confiable para la identificación del virus de influenza aviar a nivel de los locales de los mercados. En un estudio epidemiológico seccional cruzado realizado en estos mercados de venta de aves en pie se demostró que en los pasados 12 meses los mercados que permanecieron abiertos durante los siete días de la semana, y aquellos que también vendían conejos, fueron los que presentaron los niveles más altos de muestras positivas para la presencia del virus. Aquellos mercados que cerraban durante uno o más días de la semana y aquellos que realizaban limpieza y desinfección de los locales diariamente presentaron los niveles más bajos de muestras positivas a la presencia del virus.

Key words: avian influenza virus, retail live-bird markets, RRT/PCR assay, risk factors

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Abbreviations: AIV = avian influenza virus; CEAH = Center for Epidemiology and Animal Health; LBMs = live-bird markets; NJ = New Jersey; NVSL = the National Veterinary Services Laboratories; NY = New York; OR = odds ratio; p value = probability; RRT/PCR = real time reverse transcriptase/polymer chain reaction assay; SEPRL = Southeast Poultry Research Laboratory

Low-pathogenicity H7N2 avian influenza virus (AIV) has been isolated repeatedly since 1994 from retail live-bird markets (LBMs) in the northeastern United States. Presence of H7N2 virus in the LBMs poses a significant and continual risk to the commercial poultry industry in the region. Additionally, the virus has undergone several genetic changes at or near the hemagglutinin cleavage site that could lead to an increase in virulence of the virus if left to circulate, unabated, in the LBMs (8). Therefore, state regulatory officials have been attempting to rid the markets of low-pathogenicity H5 and H7 AIV. In 1999, the U.S. Department of Agriculture established a live-bird market working group to provide support to the states in developing a plan to eliminate the H7N2 virus from the LBMs in the northeastern United States. Recommendations from the working group included the validation of a real time reverse transcriptase/polymer chain reaction assay (RRT/PCR) to detect H5 and H7 subtype AIVs in LBMs and conduct an epidemiologic study to identify possible risk factors for low-pathogenicity avian influenza entrance to and maintenance in the retail live-bird marketing system. The epidemiologic study was designed in two phases: phase 1 examined the LBMs and phase 2 surveyed suppliers to the LBMs. Phase 1 was designed as a cross-sectional cohort study to identify risk factors for presence of H5 and H7 AIV in LBMs and to determine the number of markets positive for H7N2 AIV. Samples collected during phase 1 were also used to validate the RRT/PCR for detection of AIV in LBMs. Phase 2, a descriptive study, will be reported elsewhere (1).

MATERIALS AND METHODS

Epidemiological study. From July 16 to August 10, 2001, a questionnaire was administered and samples taken at all (109) live bird markets in New Jersey (NJ) and New York City (NY). Sample size was limited by the number of LBMs in operation in the study area. It was estimated that by testing all 109 markets, an odds ratio of 3–4 with 90% confidence could be attained. Acceptable P value was set at 0.05. Characteristics (risk factors) that were shared by fewer than 20 markets were not examined, since the differences would not be large enough to detect with testing methods used in the study. Sampling rates were chosen with a 95% confidence of detecting at least one infected bird assuming a 10% within-market AIV prevalence in infected markets. The within-market detection rate was based on surveillance testing results in NY and NJ LBMs since 1994.

The questionnaire was designed to identify possible risk factors for the maintenance of AIV in LBMs as suggested by the live-bird market working group. These factors included the state where the LBM was located (NY has defined sanitation and AIV testing requirements, at the time of the study NJ did not), type of birds present, length of time birds were in the market, number and types of bird sources, number of days per week the market was open/closed, whether or not newly introduced birds were placed in cages with birds already present in the market, presence/absence of wild birds in the market, accepting birds from other markets, presence of an avian mascot in the market, cleaning and disinfecting practices (frequency, products used, method, vehicles), dust buildup, presence of rodents, type of ventilation system, presence of mammals (especially red meat areas), method of handling dirty empty crates, types of cages, and waterers used. Questionnaires were reviewed and callbacks made to verify data when necessary.

Sample collection and environmental monitoring. Concurrent with questionnaire administration, samples were collected for virus isolation and the environmental conditions in the LBMs were recorded. In general, 50 bird swabs (25 tracheal and 25 cloacal) were collected per market by sampling 25 birds. Only cloacal swabs were collected from waterfowl; therefore, additional waterfowl or other birds may have been sampled to achieve the 50 sample minimum. Fewer than 50 bird samples were collected at some markets because of lack of birds. The range for the number of birds sampled was 0 to 35. In one market, no birds were present at the time of the study and only environmental samples were collected. Swabs were pooled by lot, type of bird, and sample type (cloacal, tracheal, or environmental), up to five per tube in approximately 2 ml aliquots of brain heart infusion broth.

Selecting birds for sampling was based on the following criteria, in order from highest to lowest priority: 1) lots present in the market for 1 to 5 days, (a lot was defined as a group of birds of the same type that

arrived from the same supplier on the same date), 2) birds from two large (>80 birds), two medium (20–80 birds), and two small lots (<20 birds), 3) random selection from remaining lots to capture the variety of birds present in the market, 4) sick looking birds, and 5) lots with fewer than five birds. Pigeons were not tested. When possible, bird sources were verified by viewing a receipt. (Receipts are required to be kept at the markets in NY; this was not required in NJ at the time of the study.)

Environmental samples were collected as follows: five swabs each from wet and dirty areas such as water troughs, floors, and drains in bird areas; the bird slaughter area; and the office and/or waiting room area, if present. Ten additional environmental swabs were taken from red meat areas, if present.

Samples were collected, packed with frozen gel packs, and shipped daily by overnight courier to the Southeast Poultry Research Laboratory (SEPRL), Athens, GA, for RRT/PCR testing (7). At SEPRL, an aliquot of each pool sample was aseptically removed and the remaining sample was again packed with frozen gel pack and shipped by overnight courier to the National Veterinary Services Laboratories (NVSL) for virus isolation attempts (5,6). Therefore, pooled samples for virus isolation generally arrived at the NVSL within 48 hr of collection. Test results generated at each laboratory were sent to the Center for Epidemiology and Animal Health (CEAH) for analysis with SAS software.

Results obtained from virus isolation studies were used for analysis of risk factors. A positive market was defined as a market in which at least one tube had H7 or H5 virus isolated. However, since only two markets were positive for H5N2, risk factor analysis was based only on H7N2 positive markets. Isolation of AIV subtypes other than H7 were recorded but not factored into the risk analysis. Results for RRT/PCR were recorded as positive for AIV (any subtype), positive for H7, or positive for H5. To eliminate bias in test results, the laboratories were unaware of each other's results until all test results for the study were completed.

Ambient temperature, humidity, and CO_2 measurements were taken at all LBMs. Measurements were made immediately outside the market as well as inside the bird area. Measurements were obtained with a TSI 8551 IAQ meter (Industrial Environmental Monitoring Instruments Inc., Columbus, OH). Environmental factors were measured to determine the risk from high market humidity and poor ventilation (CO_2 levels were used as a measure of air exchanges).

RESULTS

Results of surveillance in LBMs in 2001 showed that AIV was isolated from 303/1573 (19.3%) sample pools and 65/109 (59.6%) markets (Table 1).

Table 1. Comparison of the frequency of isolation of avian influenza virus from samples and live-bird markets.

Condition	No. positive	No. negative	Total tested
H7N2 (tube level)	296	1277	1573
Any AIV (tube level)	304	1269	1573
H7N2 (market level)	62	47	109
Any AIV (market level)	65	44	109

More specifically, the H7N2 virus was isolated from 296 (18.8%) sample pools and 62/109 (56.9%) markets. The unadjusted percentage of positive markets in NY and NJ was similar at 61.7% and 50%, respectively. In addition to the H7N2 subtype, AIV H5N2 was isolated from two LBMs, one each from NY and NJ. The NY market was also positive for H7N2. The H5 and H7 viruses were characterized as low pathogenicity by the chicken pathogenicity test and deduced amino acid sequence at the cleavage site of the hemagglutinin protein.

In the LBMs, H7N2 AIV was isolated most frequently from spent white fowl and guinea fowl with isolation rates of 47.8% ($n = 23$) and 32.5% ($n = 160$), respectively (Table 2). In nine H7N2-positive markets, the virus was only isolated from birds other than chickens. In these markets, H7N2 virus was isolated from guinea fowl (seven markets) and turkeys (two markets). In no market was the virus isolated from only waterfowl. The H7N2 virus was detected from at least one tracheal swab in 61/62 markets by both virus isolation and RRT/PCR.

Results used in calculating sensitivity and specificity of RRT/PCR are presented in Tables 3 and 4. At the market level the RRT/PCR had a sensitivity of 91.9% and specificity of 97.9% in detecting the H7N2 virus when compared to virus isolation. At the tube level the sensitivity and specificity was 65.8% and 96.9%, respectively.

Market characteristics, as determined by the epidemiologic study, were analyzed at several levels. Initially, each odds ratio (OR) and probability (P value) was analyzed univariately. The characteristics were then analyzed controlling for the size of the market (more or less than 300 birds present) and the number of days open (7 days per week *vs.* fewer than 7 days per week). Controlling (modeling) for these two variables accounted for the majority of the difference in prevalence between states. The following characteristics were found, based on OR and P values from the modeled analysis.

Table 2. Percent sample pools (tubes) yielding H7N2 avian influenza virus by bird type.

Bird type	Number of sample pools (tubes)	Percent sample pools positive (H7N2)
Chickens (all types)	851	22.4
Waterfowl (all types)	143	7.7
Game birds[A] (all types)	33	21.2
Guinea fowl	160	32.5
Turkeys	29	20.7
White broilers	239	18.8
Large white broilers (roasters)	10	
Red broilers	124	29
Black broilers	2	
Pheasant	1	
Spent red fowl	169	16
Spent white fowl	23	47.8
Rock (gray broilers)	181	27.6
Muscovy duck	60	15
Pekin duck	11	
Other duck	69	2.9
Bantam	4	
Silkies	25	
Quail	22	18.2
Chukar partridges	10	
Peafowl	0	
Roosters	65	26.1
Other chickens	9	
Geese	3	

[A]All game birds for this study are pheasant, quail, chukar partridge, and peafowl.

Increased risk of finding H7N2 in a retail market. See Table 5 for odds ratio, *P* values, and confidence intervals. 1) Number of days open per week (being open 7 days per week was always highly significant, even when modeled with every other variable), and 2) presence of rabbits in the market during the last 12 months.

Decreased risk of finding H7N2 in a market. 1) Being closed one or more days per week, and 2) cleaning and disinfecting daily (44% of markets that disinfect daily were positive *vs.* 73% of markets that disinfect weekly)

Marginally significant risk ($0.05 < P < 0.1$). 1) Having more than 300 birds present in the market and 2) adding new birds to cages with existing birds.

Not found to be significant risk. 1) Presence or signs of rodents, 2) number or types of suppliers used, 3) state where the market was located, 4) presence of livestock (cattle, pigs, sheep, and goats), 5) storage of dirty, empty crates in the market, 6) number of days birds remain in the market, 7) humidity level in the market, and 8) poor market ventilation (as measured by CO_2 levels). In addi-

Table 3. Sensitivity (194/295 = 65.8%) and specificity (1216/1255 = 96.9) of real time reverse transcriptase/polymer chain reaction (RRT/PCR) assay to detect H7N2 avian influenza virus at the tube level compared to virus isolation (VI).

	RRT/PCR negative (H7)	RRT/PCR positive (H7)	Total tested
VI negative	1216	39	1255
VI positive	101	194	295
Total	1317	233	1550

Table 4. Sensitivity (57/62 = 91.9%) and specificity (46/47 = 97.9%) of real time reverse transcriptase/polymer chain reaction (RRT/PCR) assay to detect H7N2 avian influenza virus at the market level compared to virus isolation (VI).

	RRT/PCR negative (H7)	RRT/PCR positive (H7)	Total tested
VI negative (H7)	46	1	47
VI positive (H7)	5	57	62
Total	51	58	109

Table 5. Odds ratio (OR) and *P* values for significant and possibly significant risk factors related to the presence of H7N2 virus in live-bird markets.

Characteristic	*n*	% positive	Univariate OR	Univariate *P* value (adj.)	Modeled OR	Confidence interval	Modeled *P* value
<7 days open per week	36	36.1	0.28	0.003	0.29	0.12–0.70	0.006
Rabbits present w/in 12 months	77	67.5	4.6	0.001	4.1	1.6–10.4	0.004
Clean and disinfect daily	41	43.9	0.42	0.04	0.39	0.16–0.95	0.04
<300 birds present	42	42.9	0.43	0.04	0.47	0.20–1.1	0.08
Add new birds to cage with old	80	63.7	2.9	0.02	2.3	0.84–6.2	0.1

tion, wild birds loose in the market was not a risk factor.

DISCUSSION

The sensitivity and specificity of the RRT/PCR procedure as found in this study suggests the procedure would be useful as a rapid screening test to identify H7 infected markets. Because of low pooled sample (tube level) sensitivity, the procedure would be less useful at identifying individual lots of positive birds.

Results of this study also confirm that, for this strain of H7N2 virus, tracheal samples yield virus more often than did cloacal or environmental samples. All but one positive market had at least one tracheal tube that yielded the H7N2 virus. The ability to detect an infected market during surveillance screening may also be enhanced by preferential testing of chickens, guinea fowl, and game birds since these species showed the highest virus recovery rates. No H7 positive markets were detected based on positive waterfowl samples alone. No specific reason could be determined for the high percentage of positive spent white laying fowl. Seven positive lots of spent white fowl were from five different sources and had been present in tested markets for greater than 24 hr. Results of this study also confirm that, for this H7N2 virus, the most efficient use of environmental sampling may be to test the bird environment and bird kill areas.

Market size was selected for modeling because it is a broad descriptor; differences among large and small markets could be vast. Size of the market (more or less than 300 birds present) was based on the number of birds present in the market at the time and day of the survey. This may have resulted in some misclassification bias, since some markets sell out of birds or almost out of birds by the end of the day. Thus, a market visited at 8:00 am may have had 400 birds, but by 5:00 pm may have only 50 birds. Significance of the size of the market is discussed below. To give a better overview of the impact of size, future surveys should ask the maximum, minimum, and typical number of birds present in the market per day.

Number of days open per week was a significant factor for being H7N2 positive. This factor was significant no matter with which variable it was modeled. This significance was not found with other factors that might be linked to being closed, such as having days empty of birds ($P = 0.17$). However, the questionnaire asked only if the market *ever* had days empty of birds, not how often or regularly this occurred. Markets with occasional empty days would have answered "yes" to having days empty of birds, yet may have been open 7 days per week normally. Only eight markets reported being empty of birds when disinfected (*n* too small to analyze).

The significance of disinfecting the market daily versus weekly or less often may have been biased by several factors. The questionnaire defined disinfection as "clean first with a detergent cleaner, removing all manure, dust, etc. before applying a disinfectant." However, some interviewers/market owners may have interpreted this as the entire market (cages, walls, etc.) while others may have interpreted it to mean any area of the market (e.g., floors daily). If the interviewer noted on the questionnaire that only portions of the market were disinfected daily, but a complete disinfection was done less often, the questionnaire was coded for less than daily. Additionally, the answers may have been biased by the Hawthorne effect. These types of bias tend to drive the significance toward the null. Therefore, disinfecting daily may be more protective against H7 infection than indicated by this study.

The presence of rabbits in the market greatly increased (OR = 4.1, $P = 0.004$) the risk of a market being found positive for H7 AIV. Little research on the role of rabbits in avian influenza transmission exists. The increased risk could be due to some undetermined factor regarding the rabbits them-

selves, or it could be because markets that sell rabbits differ from markets that do not in some as yet undefined factor. One possible explanation is suppliers who deliver rabbits may also supply higher risk (untested) birds. However, in poststudy survey of NJ LBMs, rabbits were reportedly obtained from wholesalers that also supply tested birds. The sources of rabbits to the markets should be further investigated.

It is biologically plausible that the risk of AIV infection would be increased by the factors for which the sample size may have been too small to detect a difference ($0.05 < P < 0.1$). As discussed above, misclassification bias in reporting the size of the market could have affected the significance of this factor. Economic analysis of the markets done in preparation for market testing and closure determined little difference in the number of birds sold daily at each market (three of four markets sold $1,400–$2,758 per week) (3). It may be that small markets sell out or almost out of birds daily, receiving new bird shipments frequently. Larger markets may have more leftover birds, receiving less frequent shipments of new birds. This would leave infected birds in the market to transmit virus to new arrivals. Although the reported mode time that lots of birds had been in the market was the same for positive and negative markets (1 day) and the range was fairly similar, (positive markets 1–6 days, negative markets 1–4 days) these numbers may not reflect the true time extremes that birds may remain in a market. Lack of detection of an effect of days in the market may be due to inaccurate reporting of the date the birds were received, even if verified with a receipt. For example, white broilers indicated as having arrived today may, in fact, be a cage of just received broilers added to a cage of broilers received previously.

Placing newly arrived birds in cages with leftover birds is also a logical risk for avian influenza infection. Birds can become infected and shed avian influenza after less than 24 hr in an infected market (2,4). If newly infected but leftover birds are held overnight, they may spread the virus to new birds the next day. Owing to this commingling of birds, if the market is not regularly empty a bird reported as having been in the market one day may in fact have been there for a week or longer. Significance of mixing of birds may have been biased towards the null because market owners may report what they think the interviewers wanted to hear (Hawthorne effect). However, only 29 markets reported never mixing birds.

Phase 2, a descriptive study of suppliers to the New Jersey and New York LBMs, was completed in November 2001. A supplier database was compiled for the study from sources identified by the live-bird retail markets, sources known to federal and state personnel (including wholesalers and approved poultry dealers or haulers), frequent poultry auction buyers, and producers testing for AIV (regularly or sporadically). It is likely this database did not include all suppliers to the LBMs. Of the 2225 sample pools collect from suppliers in the phase 2 study, no H7 or H5 AIV was isolated. The two studies (phases 1 and 2) support the theory that AIV is maintained within the LBMs and may be sporadically reintroduced from unapproved/unknown supply sources.

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